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ANNUAL PROGRESS REPORT III
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I. INFLUENCE OF TOTAL-BODY X-RAYS ON THE KINETICS OF ERYTHROCYTE ENZYMES AS A BIOLOGICAL DOSIMETER
II. BIOCHEMICAL STUDIES OF THE RAT BRAIN

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ABSTRACT

An internal X-ray dosimeter has been found in a rat erythrocyte enzyme, nucleoside phosphorylase. At all total-body X-ray doses tested from 100 to 1000 r, this enzyme is inhibited but the rate of inhibition is closely similar for the 250, 500, 750, and 1000 r in the postirradiation interval from 0 to 48 hours. However, the time and degree of recovery of enzyme activity postirradiation is dose dependent. Thus, complete recovery after 100 r was in 116 hours, after 250 r in 155 hours, and after 500 r in 221 hours. With a 750 r dose only partial recovery of activity was noted at 216 hours, but at 1000 r dose there was no recovery of enzyme activity. The inhibition of this enzyme after 100 r was 31 percent in 48 hours, after 250 r 59 percent in 72 hours, after 500 r 73 percent in 96 hours, after 750 r 75 percent in 96 hours, and after 1000 r 76 percent in 96 hours. In the immediate postirradiation interval of 16 hours, 100 r total-body X-irradiation of the rat inhibited erythrocyte nucleoside phosphorylase 14 percent and after 500 r the inhibition was 36 percent.

A microtechnique has been developed and initiated for testing the erythrocyte nucleoside phosphorylase activity which will permit repeated sampling of blood from rats and other subjects. This will permit using each animal as its own preirradiation control and the same animal will also be used for multiple sampling of blood throughout the postirradiation period.

Arrangements have been initiated to test the total-body X-irradiation effect on nucleoside phosphorylase in erythrocytes from monkeys. Preliminary studies on human subjects are planned.

Studies on the subcellular metabolism of the rat brain were continued. Use of nicotinamide in brain mitochondrial preparations revealed that it not only prevented the hydrolysis of pyridine nucleotide coenzymes (NAD and NADP), but it also affected the oxidative metabolism of isocitrate.

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4. IV. Effect of Nicotinamide on Brain and Liver Mitochondria, M. R. V. Murthy and D. A. Rappoport, Biochimica et Biophysica Acta, 1963 (in press).

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INTRODUCTION

Eight years ago, studies were initiated in our laboratories to find an internal mammalian X-ray dosimeter using the rat as the experimental organism. In a publication by Rappoport and Sewell (1) (copy enclosed in this report), it was rationalized that the enzymes in the erythrocytes were the most suitable body component for such studies since the erythrocytes (a) had a relatively long life span, (b) were incapable of enzyme resynthesis, and (c) can be easily sampled without trauma to the organism and without affecting the enzymes to be tested. It was also shown at that time that total-body X-ray doses of 500 and 1000 r, respectively, inhibited the metabolism of purine nucleosides, namely, guanosine, inosine, and adenosine. It was demonstrated that these nucleosides served as a source of pentose which was the substrate for a sequence of enzymatic reactions which yielded a variety of phosphate esters and lactic acid. It was then necessary to show what particular enzyme reaction in the sequence was inhibited by the X-ray treatment of the rats.

The present report summarizes the findings that (a) the rat erythrocytes enzyme can serve as an internal dosimeter, (b) the only enzyme in the entire sequence of purine nucleoside metabolism which was specifically affected by the total-body X-irradiation of the rat was the erythrocyte nucleoside phosphorylase, and (c) the internal dosimeter in the rat erythrocytes is not the inhibition of this enzyme, but in the recovery of the enzyme activity postirradiation which is definitively dose dependent.

It can be stated that the initial purpose of these investigations, that is, finding an internal mammalian dosimeter, has been attained. There remains the studies to be done on irradiated and non-irradiated primates as a systematic approach to the assessment of this internal dosimeter for human application. Toward these ends, arrangements are in progress to use the primate colony of the Air Force School of Medicine, Brooks Air Force Base, Texas, and a micromethod of assay for this enzyme has been devised to permit sampling 0.4 ml. of fresh blood for the assay of this enzyme so as to allow extensive tests on a single primate before and also after irradiation, thus the animal will be its own control. This procedure will greatly minimize the number of primates needed for these experiments without sacrificing any precision in the study.

This report summarizes (a) the evidence that nucleoside phosphorylase in the rat erythrocytes is the only enzyme affected by total-body X-irradiation of the rat, (b) the relationship of X-ray dose to this enzyme inhibition and subsequent reactivation, and (c) presents some data on the normal variations of this enzyme within each age group and the variations in several age groups.

EXPERIMENTAL

Radiation and lysate preparation

Sprague-Dawley rats were total-body irradiated with a G-E Maxitron X-ray generator (250 KV, 30 ma, filters were 1/4 mm. Al) at a distance of 55 cm. from the horizontal middle line of the animal.

These rats were bled by means of a heart puncture using heparinized syringes. Individual rats were bled in a group and the whole blood was pooled in equal volumes from each animal within a group. The blood was cooled and the erythrocytes were separated by centrifugation. The plasma and white cell layer were removed by suction. The erythrocytes were washed three times in succession with two or three volumes of cold isotonic KCl. After each centrifugation, the saline washings were removed from the packed erythrocytes by suction. The washed erythrocytes were hemolyzed at room temperature with four volumes of glass distilled water. The resulting 20 percent hemolysate was kept at room temperature and pipetted immediately into the reaction vessel to avoid hemoglobin crystallization. Simultaneously, aliquots were taken for dry weight determinations.

The incubation medium contained 2 umoles of guanosine, 0.05 M phosphate buffer (pH 7.4) and lysates of various concentrations in a total volume of 1.0 ml. The reaction mixtures were incubated in air at 37°C, and terminated after 0, 10, 20, and 30 minutes by the addition

of 0.5 cc of cold 15 percent perchloric acid. The supernatant was separated from the residue by centrifugation and aliquots were taken for guanine-xanthine analysis. The combined concentration of guanine and xanthine was determined by the colorimetric method described by Hitchings (2).

The aliquots taken from the deproteinized supernatant were made up to a volume of 1 ml. with glass distilled water. Two ml. of 20 percent sodium bicarbonate and 2.5 ml. of glass distilled water was added. Shaking was necessary during and after the addition of phenol reagent to insure reliable results. After the addition of the phenol reagent, the color was allowed to develop at 37°C for a period of 20 minutes and the color intensity was measured at 660 mu using a DU-Beckmann spectrophotometer.

Investigations of enzymes affected by X-rays

The animals were divided into eight groups with two rats in each group. Four groups served as non-irradiated controls, while the remaining four groups were irradiated at 770 r total-body. A group of irradiated and unirradiated control animals were sacrificed simultaneously at intervals of 21, 45, 69, and 93 hours postirradiation. In the analyses of the following enzymes, hexokinase, aldolase, enolase, pyruvate kinase, glucose-6-phosphate dehydrogenase, lactic dehydrogenase, and 6-phosphogluconate dehydrogenase, it was necessary to

remove the ghost and freeze the hemolysate over night to insure crystallization of a major portion of the hemoglobin. The high hemoglobin concentration interferred with the UV absorption at 340 mu where most of the above enzyme activities were measured for the formation of NADH or NADPH. The crystallized hemoglobin was separated by high speed centrifugation (30,000 x g for 1 hour).

Inosine as a substrate and biological variations among individual rats

Four groups of rats, each group consisting of 24 animals, were irradiated at 0, 250, 500, and 750 r total-body and the enzyme activity was measured at 48 hours postirradiation. The erythrocytes from each animal were analyzed separately to determine the biological variation in the control as well as the irradiated rats. The procedure for the preparation of lysate was the same as described above, but the final lysate concentration used in these incubations was 7.5 percent. The incubation medium contained 2 umoles of inosine, purified xanthine oxidase (1500 units), purified catalase (1000 units), and 0.01 M phosphate buffer (pH 7.4) in a total volume of 1.0 ml. Utilization of inosine was determined by the differential pentose analyses described in prior reports (Progress Report I, September 1961 - August 1962).

Late postirradiation effects

Groups of fifteen rats each (275-325 g) were irradiated at 0, 100, 250, 500, 750, and 1000 r total-body and, subsequently, three rats

were sacrificed at each period 48, 96, 144, 216, 264 hours postirradiation, and the erythrocytes were assayed for nucleoside phosphorylase activity. With each irradiated group, three unirradiated controls were also sacrificed and their erythrocytes assayed.

Early postirradiation effect

Groups of 30 rats each (175-225 g) were irradiated at 100 and 500 r total-body and groups of six rats were sacrificed at 4, 8, 16, 24 and 48 hours postirradiation.

Age versus activity of nucleoside phosphorylase

An age study was initiated since unusual results were obtained by the irradiation of younger rats (175-225 g) as compared with the older rats (250-300 g) where a higher activity of nucleoside phosphorylase was found in the erythrocytes from the younger rats. A group of ten 32 day old rats (75 g each) and groups consisting of five rats in each of 46, 62, 73, and 93 day old rats were bled individually and the whole blood from each group was pooled in equal volumes and processed as described above. The final lysate concentrations in the incubation of the 32, 46, and 62 day old rats were 2 and 4 percent, and of the 73 and 93 day old rats were 4 and 8 percent. The average weights were as follows:

32 day old rat		75 g	average weight per rat
46 " " "		155 g	" " " "
62 " " "		235 g	" " " "
73 " " "		275 g	" " " "
93 " " "		385 g	" " " "

Micromethod for nucleoside phosphorylase assay

The rat was anesthesized with ether and the tail vein was punctured with a blood lancet. Approximately 0.4 ml. of blood was removed and 0.2 ml. was immediately placed into two Coleman micro-centrifuge tubes. The erythrocytes were separated from the plasma and white cell layer by centrifugation for 1.5 minutes in a Beckmann-Spinco Microfuge. The packed erythrocytes were washed three times with cold isotonic KCl and finally lyzed with nine volumes of glass distilled water. Hemoglobin was determined by mixing 20 lambda of the hemolysate in 5.0 ml. of 0.4 percent (v/v) ammonium hydroxide solution and the absorbancy was measured at 540 mu with a Beckmann DU-spectrophotometer.

The incubation medium contained 0.2 umole of guanosine, final lysate concentration of 0.4 percent, 5 umoles of phosphate buffer (pH 7.4) in a total volume of 0.1 ml. The reaction was terminated by the addition of 0.05 ml. of cold 15 percent perchloric acid at intervals of 0, 10, 20, and 30 minutes. Aliquots (0.04 ml.) were taken and mixed with 0.66 ml. of water and 0.4 ml. of 20 percent Na_2CO_3 solution. Shaking was necessary during the addition of 0.1 cc of phenol reagent. The colors were allowed to develop at 37°C for a period of 20 minutes. The samples were then immediately read in 1.0 ml. cells at 660 mu using a Beckmann DU-spectrophotometer.

RESULTS

Enzyme inhibition

When the metabolism of guanosine by rat erythrocytes was investigated following 500, 750, and 1000 r total -body irradiation, approximately 75 percent inhibition of guanosine metabolism was noted in contrast to the unirradiated controls at 96 hours postirradiation (Table I) (1). In order to establish where the inhibition occurred, the sequence of enzymic reactions illustrated schematically in Figure 1, were tested systematically, one enzyme at a time. Table II shows the results obtained. No enzyme other than nucleoside phosphorylase, the first enzyme in the scheme illustrated in Figure 1, was affected. It was thus established that the total-body X-irradiation of the rat markedly inhibited erythrocyte nucleoside phosphorylase.

Inhibition and recovery

Using the assay for nucleoside phosphorylase activity in erythrocytes, groups of rats were irradiated total-body with a single dose of 100, 250, 500, 750, and 1000 r, respectively, and enzyme assays were made at intervals of 3, 8, 16, 24, 48, 96, 144, 216, 264 hours postirradiation. Figures 2a, 2b, 3a, and 3b illustrate the results obtained. All doses affected a marked decrease in nucleoside phosphorylase activity. The earliest inhibition occurred at 16 hours postirradiation

after a dose of 100 r (Figures 2a and 2b). Maximum inhibition of 75 percent occurred at 96 hours after an X-ray treatment of 500, 750, and 1000 r, respectively (Figures 3a and 3b). Inhibition of this enzyme was not clearly dose dependent since the rate of this inhibition, following 250, 500, 750, and 1000 r, was somewhat similar.

However, erythrocyte nucleoside phosphorylase showed a distinct dose dependent recovery in those experiments in which rats were irradiated with 100, 250, and 500 r, respectively. Complete recovery of activity occurred in the postirradiation interval of 116 hours in the 100 r group, 155 hours in the 250 r group, and 221 hours in the 500 r group (Figure 4). Enzyme activity in the 750 r group showed a trend toward recovery in 144 hours, but this activity persisted at 57 percent below the unirradiated controls at 216 hours postirradiation. Erythrocytes enzyme from 100 r treated rats remained at the 80 percent below the controls and did not show any tendency toward recovery.

Normal variations

In replicate experiments, variation in nucleoside phosphorylase activities were noted in both erythrocytes from individual controls and irradiated rats. Table III illustrates the observed variations in activities of this enzyme using inosine as the substrate.

Influence of rat age

It was noted that erythrocytes from young rats contained a greater amount of enzyme activity than older rats (3). Table IV and Figures 5 and 6, show the activities of nucleoside phosphorylase in erythrocytes from 32, 46, 62, 73, and 90 day old rats, respectively. When young rats (age 50 days) were treated with 100 and 500 r total-body X-rays, the degree of inhibition of erythrocyte nucleoside phosphorylase activity in the young rats was the same as that noted in the erythrocytes from mature rats on the basis of percent inhibition (Figure 2a).

Inosine as a substrate

Using inosine instead of guanosine as the test substrate, inhibition of nucleoside phosphorylase is also observed following total-body X-irradiation. Figure 7 illustrates the results obtained after X-ray treatment 250, 500, and 750 r at 48 hours postirradiation. These studies were primarily intended to establish the statistical variations in nucleoside phosphorylase activity in irradiated and unirradiated rats. With the above doses the erythrocyte enzyme was inhibited 33 percent, and no dose dependence was noted. The variation as standard deviation of the mean in the controls in this study was 2.68 ± 0.24 umoles of inosine utilized per 100 mg. of protein per 10 minutes and in irradiated samples at 250 r was 1.61 ± 0.10, 500 r was 1.52 ± 0.10, and 750 r was 1.76 ± 0.10 umoles inosine utilized per 100 mg. protein per 10 minutes (Table III and Figure 7).

Micro assay of enzyme

The micro procedure for the assay of nucleoside phosphorylase has been initiated and the preliminary results appear adequate as the macro procedure using phenol reagent for guanine-xanthine analysis. Data now being accumulated will permit a definitive evaluation of this procedure.

DISCUSSION

The present investigations confirmed the working hypothesis formulated by Rappoport and Sewell in 1959 (1), that the erythrocytes from an irradiated mammal can serve as an internal dosimeter following X-irradiation. In the rat, erythrocyte nucleoside phosphorylase is demonstrably inhibited even with total-body X-ray dose of 100 r (Figures 2a, 2b, 3a, and 3b, and Table I). Examination of Figure 3a shows a dose dependent inhibition of this enzyme at doses of 100, 250, and 500 r at 96 hours postirradiation, however, enzyme inhibition of the 500, 750, and 1000 r up to 96 hours postirradiation is approximately identical.

In the recovery of enzyme activity, dose dependency is clearly evident (Figure 3a) and there is a linear relationship between dose versus enzyme recovery as illustrated in Figure 4. The latter observations establish that the nucleoside phosphorylase in the rat erythrocyte

responds to X-radiation as would be expected for an internal dosimeter at sublethal dose levels of X-irradiation (100-500 r).

Unexpectedly, it was observed that the young rat erythrocytes have a higher level of nucleoside phosphorylase activity than the erythrocytes from older rats (Figures 5 and 6, and Table IV). However, when younger rats are irradiated, the percent inhibition of erythrocyte nucleoside phosphorylase is very similar to that noted with the adult erythrocytes (Figure 2a). The nucleoside phosphorylase activity levels off after the rats reach the age of 73 days.

Early in these investigations, it was noted that individual rats, of the same age and weight, yielded erythrocytes with varying nucleoside phosphorylase activity. To establish what effect these "biological" variations may have on the dose-response observations, inosine was used as a substrate in incubations of erythrocytes from individual rats. Using 24 rats for each point in the investigation, the average deviation of the mean and the standard deviation of the mean was determined and the results are replicated in Table III. It can be seen from these variations that the degree of inhibition of nucleoside phosphorylase activity by X-ray doses of 250 to 750 r was beyond the variation in the individual observations. Using inosine as the substrate for hemolysate incubation, the inhibition of nucleoside phosphorylase following 250, 500, and 750 r total-body X-irradiation, respectively, showed no dose dependency (Figure 7).

Since the ultimate purpose of these investigations is to evaluate any internal dosimeter found for application to irradiated humans, two essential prerequisites must be met before dosimeters can be used on humans: (1) a prior demonstration that the internal dosimeter is effective in primates, and (2) that an accurate microtechnique be devised to carry out continuous sampling.

Primate tests are now being negotiated with Colonel J.E. Pickering at the School of Aviation Medicine, in order to evaluate the erythrocyte nucleoside phosphorylase as an internal primate dosimeter. Secondly, a microtechnique for assaying this enzyme has been devised and it is being evaluated at the present time. The preliminary results with the micromethod are very promising.

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3. Fritz, R.R. and Rappoport, D.A., manuscript in preparation.

TABLE I

Inhibition of Nucleoside Phosphorylase after 96 Hours Postirradiation with Guanosine as a Substrate

Dosage r	Percent Inhibition
0	0
100	28.0
250	58.5
500	73.0
750	75.0
1000	76.5

TABLE II

Enzymatic Activities in RBC (SFH) from Irradiated (A) and Unirradiated (B) Rats

Enzymatic Activities as $\triangle \overline{OD}$ per minute per gram of protein*

Group	Postirradiation Interval (hours)	Hexokinase	Aldolase	Enolase	Pyruvate Kinase	LDH	G6PDH	6PGDH
A	21	4.20	14.8	27.9	25.9	136	19.4	10.0
B	--	4.40	17.5	31.8	29.9	141	22.2	10.1
A	45	3.71	17.2	28.6	32.9	108	21.1	9.78
B	--	4.05	16.3	28.6	24.9	108	22.3	9.95
A	69	4.26	18.2	27.8	28.0	121	21.1	9.20
B	--	4.66	18.8	27.8	26.6	104	17.8	8.60
A	93	3.59	12.9	25.8	24.0	97**	15.4**	8.15
B	--	4.47	18.7	25.0	24.7	96**	15.3**	8.36

* The methods of assay of these enzymes have been described in Progress Report I (September 1961 to August 1962).

** Tissue frozen twice before analysis.

TABLE III

Variations Observed Between Individual Rats of Nucleoside Phosphorylase Activity with Inosine as the Substrate

(Each figure represents 24 individual rat erythrocyte assays.)

Incubation Time	Dosage r	Inosine (umoles utilized/100 mg. dry weight)	Average Deviation of the Mean	Standard Deviation of the Mean
10 minutes	0	2.68	± 0.70	± 0.24
	250	1.61	± 0.36	± 0.10
	500	1.52	± 0.35	± 0.10
	750	1.76	± 0.44	± 0.10
20 minutes	0	4.62	± 0.76	± 0.30
	250	2.67	± 0.47	± 0.10
	500	2.58	± 0.45	± 0.10
	750	3.02	± 0.68	± 0.15
30 minutes	0	6.02	± 0.89	± 0.24
	250	3.70	± 0.57	± 0.16
	500	3.57	± 0.51	± 0.15
	750	3.90	± 0.98	± 0.24

TABLE IV

Change in Erythrocyte Nucleoside Phosphorylase Activity in Different Aged Rats

Age (days)	Weight (grams)	Activity (umoles formed/100 mg. protein per hour)
32	76	68.0
46	154	45.8
62	234	28.7
73	275	19.9
93	385	17.8

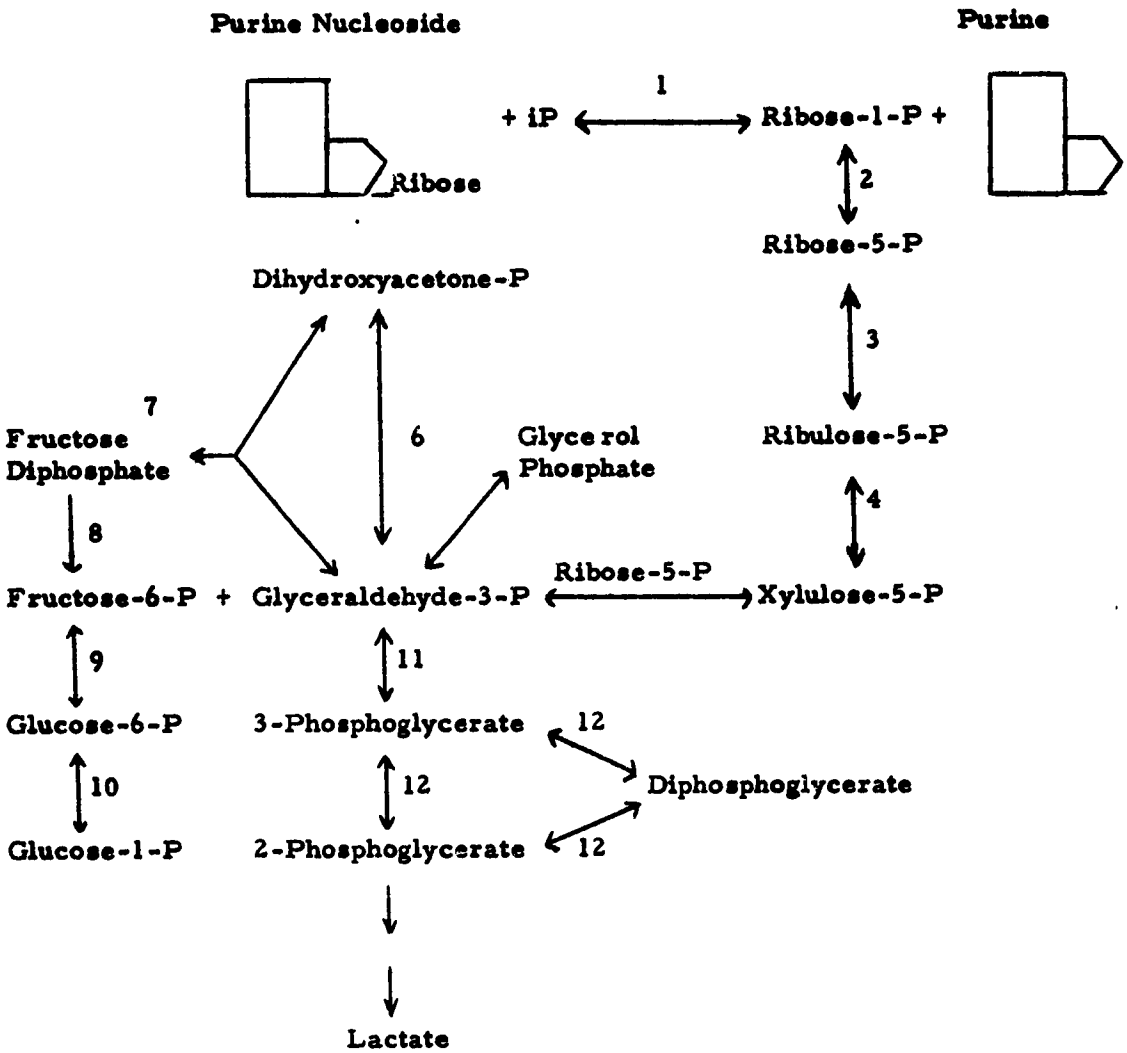


Fig. 1. Diagrammatic representation of the metabolic reactions of purine nucleosides in erythrocytes. The enzymes involved are: (1) nucleoside phosphorylase; (2) phosphoribomutase; (3) phosphoriboisomerase; (4) phosphoketopentoepimerase; (5) transketolase; (6) triosephosphate isomerase; (7) aldolase; (8) fructose diphosphatase; (9) phosphoglucoisomerase; (10) phosphoglucomutase; (11) triosephosphate dehydrogenase; (12) phosphoglyceric mutase.

Fig. 2a. Early Postirradiation Affect on Rat Erythrocytes Nucleoside Phosphorylase

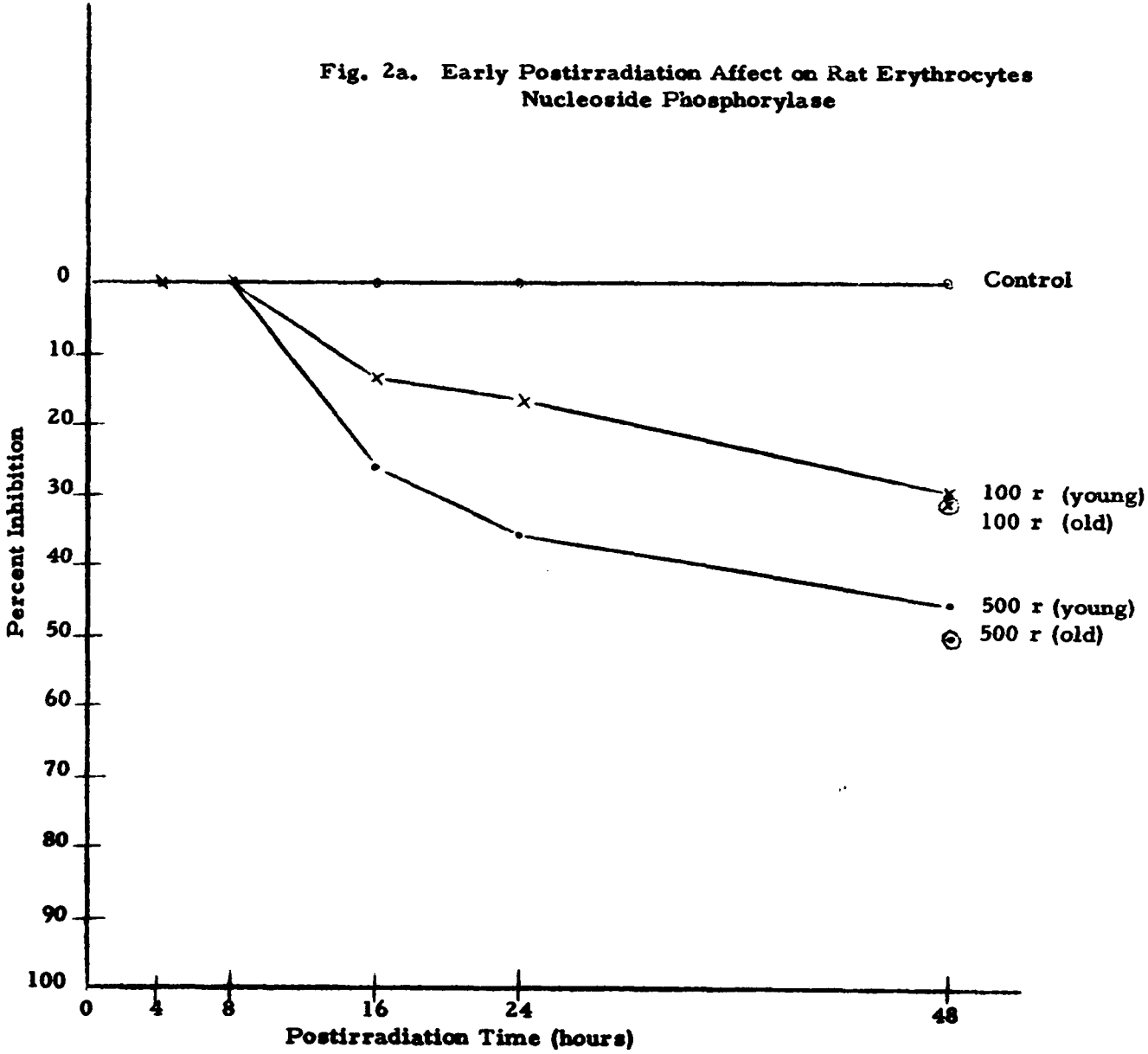


Fig. 2b. Early Postirradiation Affect on Rat Erythrocytes Nucleoside Phosphorylase

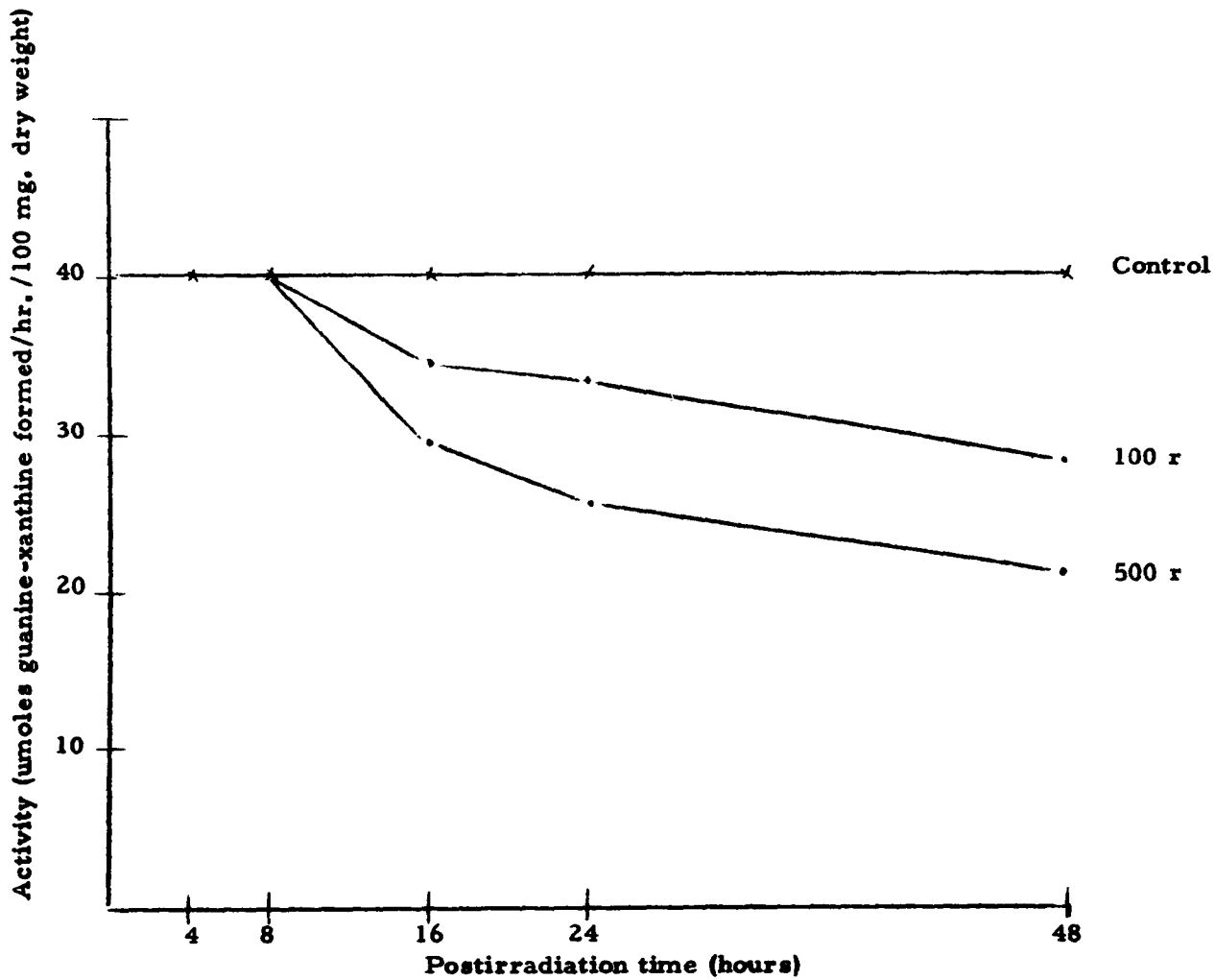


Fig. 3a. Late Postirradiation Affect on Rat Erythrocytes Nucleoside Phosphorylase

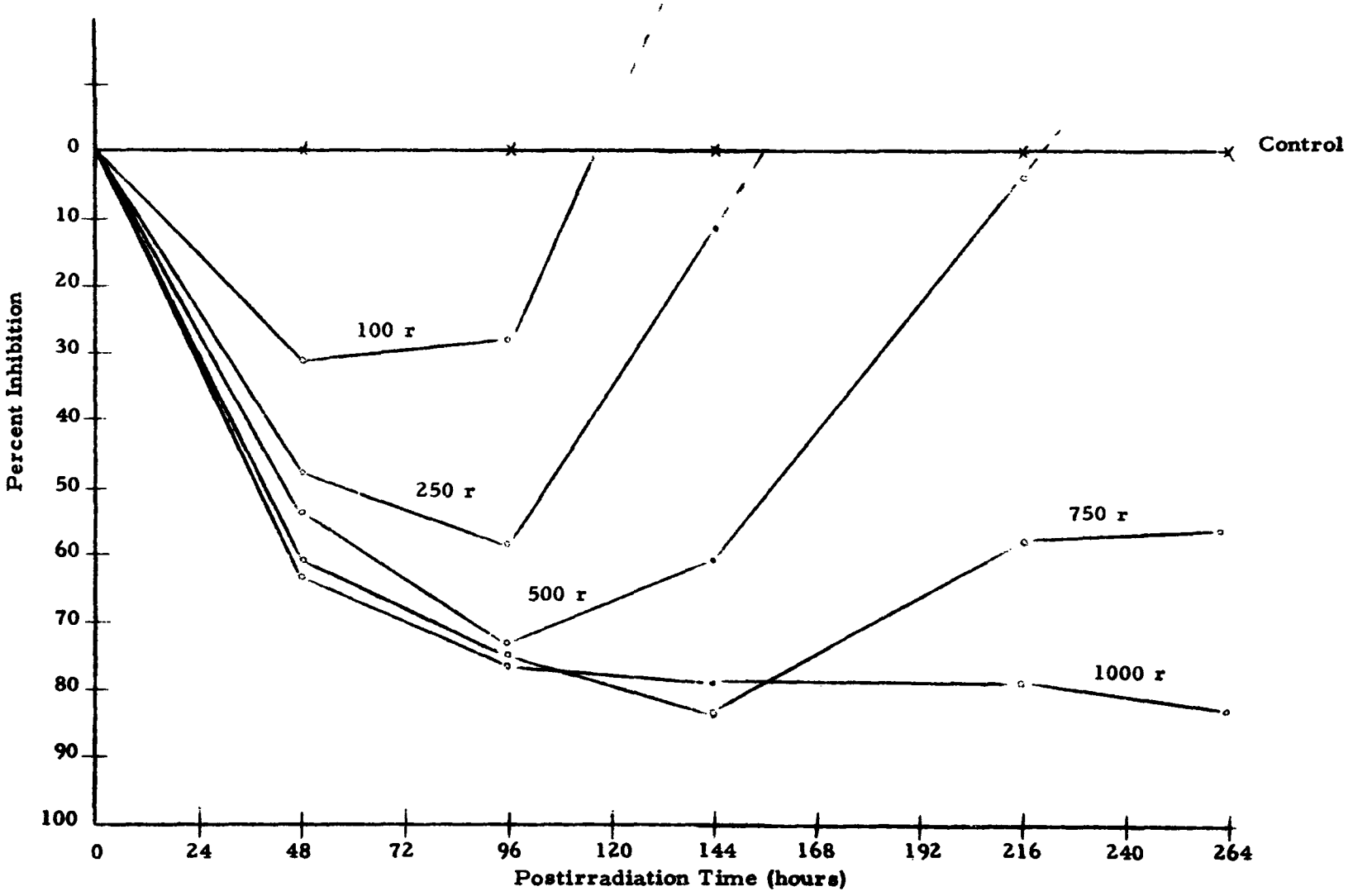


Fig. 3b. Late Postirradiation Affect on Rat Erythrocytes
Nucleoside Phosphorylase

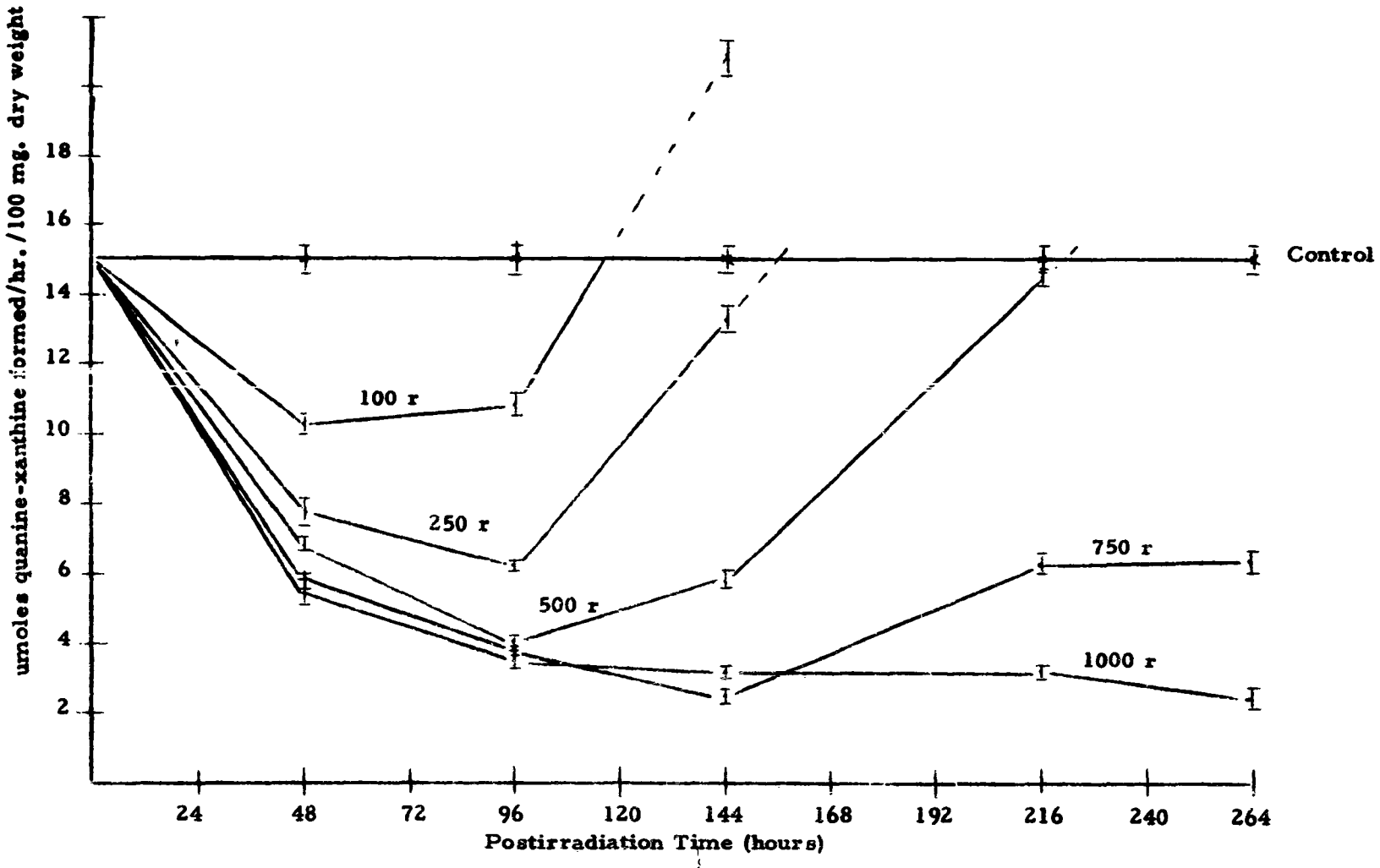


Fig. 4. Recovery of Rat Erythrocyte Nucleoside Phosphorylase Activity

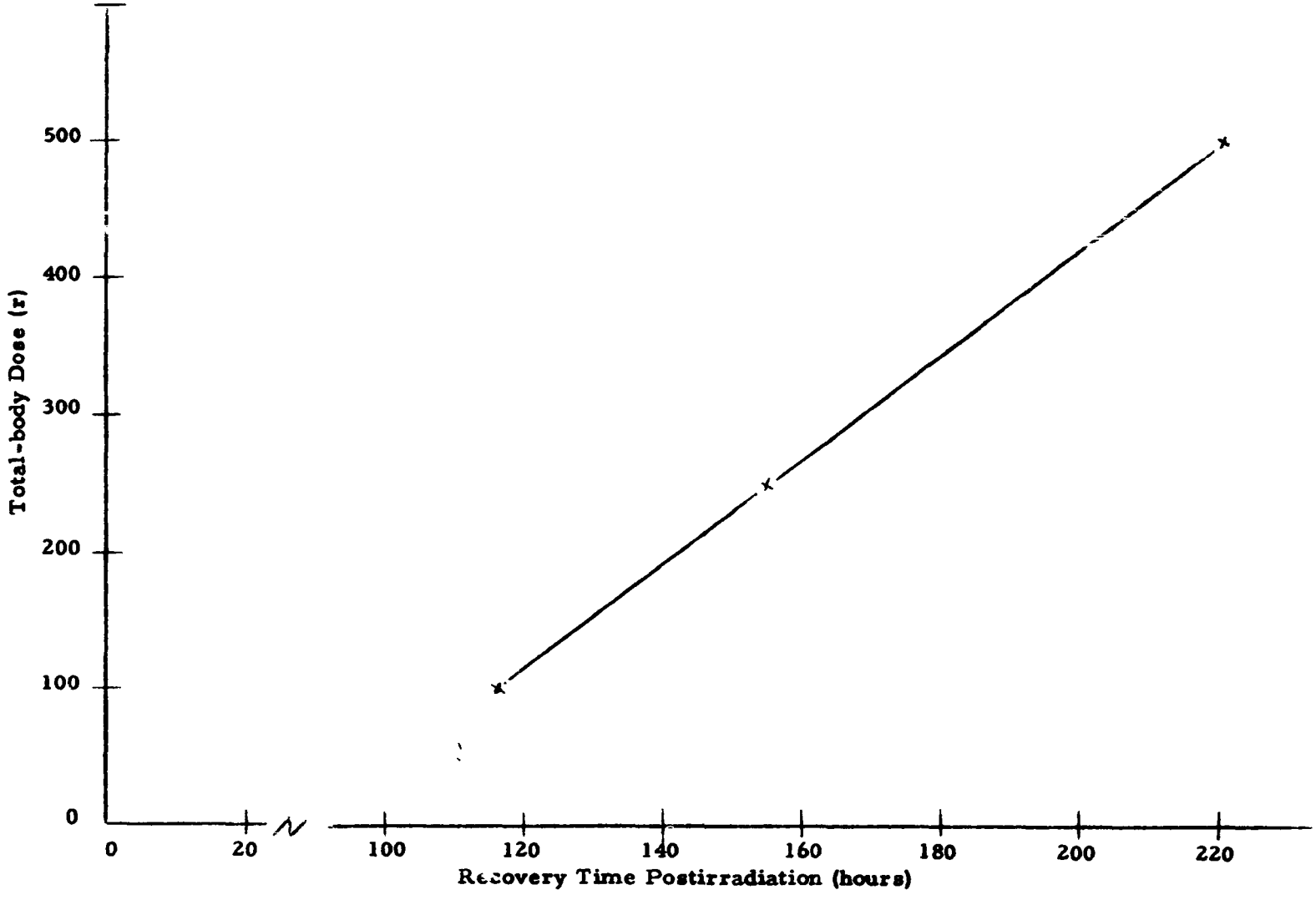


Fig. 5. The Change in Nucleoside Phosphorylase Activity in Erythrocyte from Rats of Different Ages

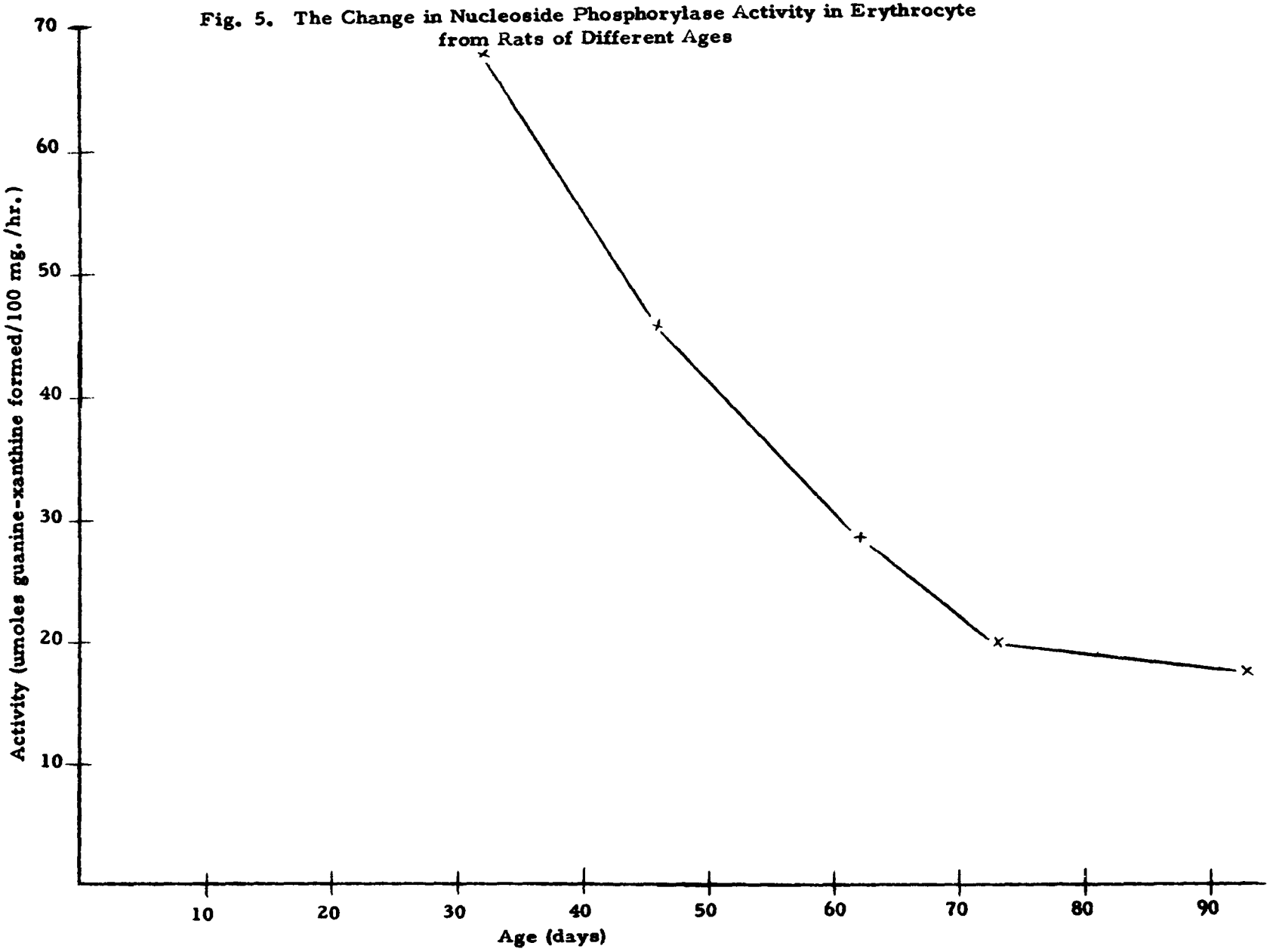


Fig. 6. The Change in Rat Erythrocyte Nucleoside Phosphorylase Activity in Rats of Different Weights

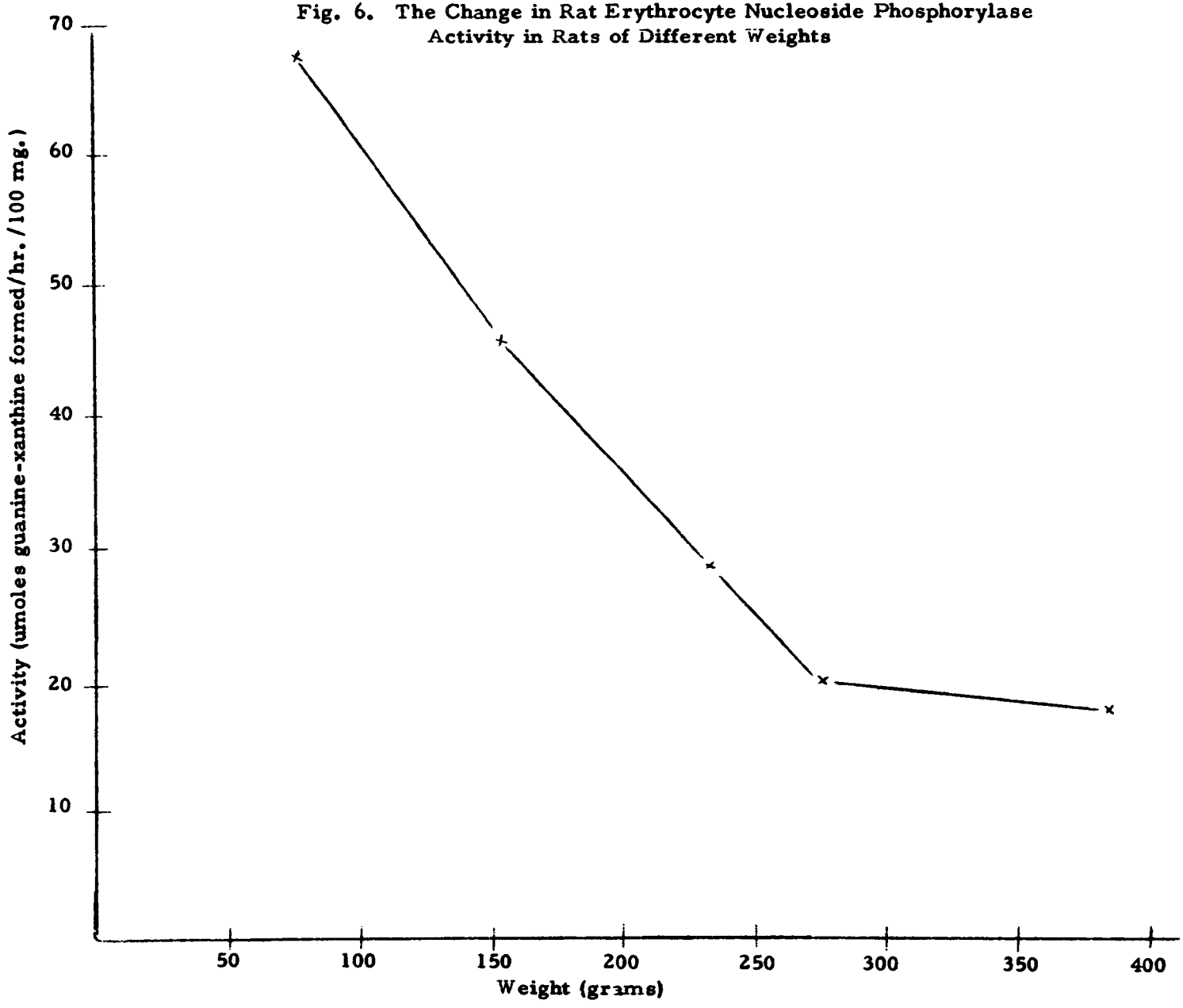
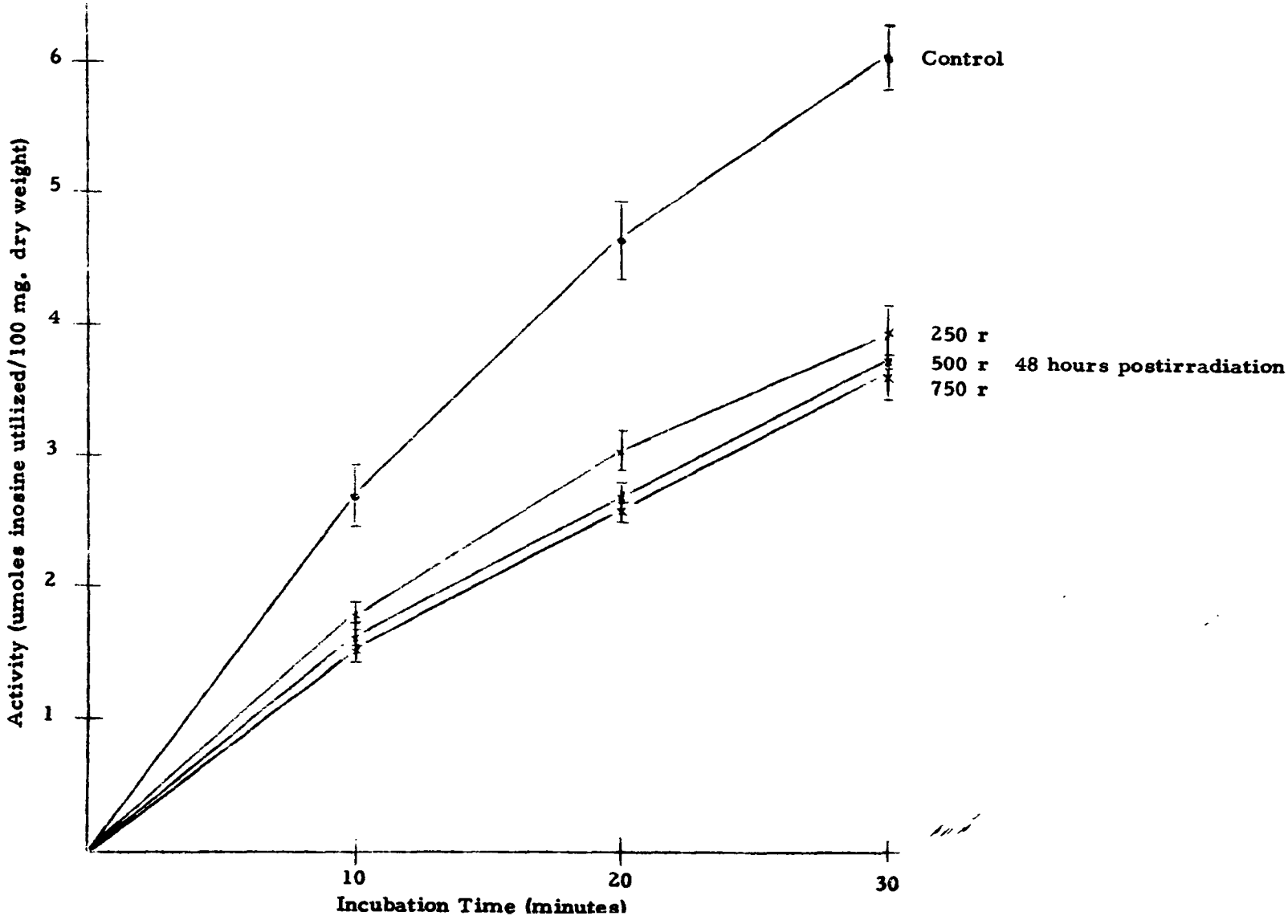


Fig. 7. Inosine Metabolism by Individual Rat Erythrocytes Samples for Determination of Individual Variation as a Standard Deviation of the Mean



BIOCHEMISTRY OF THE DEVELOPING RAT BRAIN

IV. EFFECT OF NICOTINAMIDE ON BRAIN AND LIVER MITOCHONDRIA

Abbreviations: NAD, nicotinamide-adenine dinucleotide; NADH, reduced nicotinamide-adenine dinucleotide; NADP, nicotinamide-adenine dinucleotide phosphate; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; Tris, tris (hydroxy methyl) aminomethane; ATP, adenosine triphosphate; NAm, nicotinamide.

SUMMARY

Mitochondria from neonatal and adult rat brains and also from adult rat liver were compared for their ability to oxidize isocitrate in the presence and absence of added pyridine nucleotides and nicotinamide.

Nicotinamide enhanced the oxidation of isocitrate by adult rat liver and brain mitochondria in the presence of added NAD and depressed it in the presence of added NADP. Phosphorylation associated with the oxidation of isocitrate was also affected by nicotinamide, depending on the added coenzyme and the tissue under consideration.

Nicotinamide enhanced the activity of NAD-isocitrate dehydrogenase and NADH-cytochrome C reductase of brain mitochondria. It had no effect on NADPH-cytochrome C reductase.

INTRODUCTION

The studies on the oxidation of malate, lactate and succinate by Mann and Quastel[1] led to the findings that brain extracts contained a pyridine nucleotidase which rapidly destroyed NAD and that nicotinamide protected NAD from this breakdown. Zatman et al.[2] later showed that this effect was due to an exchange reaction between free nicotinamide and bound nicotinamide which was catalyzed also by pyridine nucleotidase. That the effect of nicotinamide was not due solely to its action on NADase, was suggested by the experiments of Larner et al.[3] who found that the presence of high concentrations of nicotinamide (0.33 M) in brain homogenates resulted in an inhibition of oxygen uptake with pyruvate as substrate and this inhibition was not reversed by the addition of excess NAD; utilization of pyruvate was, however, unimpaired by nicotinamide. An explanation for these observations was afforded by the findings of Jacobson and Kaplan[4] who showed that nicotinamide inhibited NADH-cytochrome C reductases in both mitochondria and microsomes. The oxidation of citrate and isocitrate in neonatal and adult rat brain mitochondria were investigated by Murthy and Rappoport[5, 6] and preliminary experiments showed that nicotinamide enhanced the oxidation of these substrates in the presence of excess NAD.

This report deals with the effect of nicotinamide on the oxidation of isocitrate, the concomitant oxidative phosphorylation, and some of the pyridine nucleotide dependent enzymes concerned with the aerobic oxidation of this substrate. Liver mitochondria were compared to brain mitochondria, since the latter contain negligible amounts of transhydrogenase[7] and hence permitted a study of the NAD and NADP dependent dehydrogenases without mutual interference. Mitochondria from neonatal rat brain were also tested since the NADase activity in the neonate is very low[8].

METHODS

Male Sprague-Dawley rats, 1 day old (neonatal) and 8-10 weeks old (adult), were used. Except when specifically noted, the words "liver" and "brain" are used to denote adult rat liver and adult rat brain. Methods for the preparation of mitochondria and the analytical procedures have been described in previous reports[5,6].

RESULTS

Oxidation in intact mitochondria

Nicotinamide increased the oxidation of isocitrate in the absence of any added pyridine nucleotides or in the presence of added NAD (Table I). However, in the presence of added NADP, nicotinamide had either no effect or caused a decrease in oxygen uptake. When both NAD

and NADP were added, nicotinamide increased oxygen uptake although this increase was not proportionately as great as in the presence of NAD alone.

Mitochondria from neonatal brain oxidized citrate maximally only in the presence of added pyridine nucleotides, similar to the adult brain mitochondria. But in contrast to the latter, isocitrate oxidation by neonatal mitochondria remained unaffected by nicotinamide either in the presence or absence of any added pyridine nucleotides.

Since nicotinamide is known to inhibit NADase[2] and since NADase activity is very low in the neonatal brain and reaches a maximal value in the adult[8], it appeared that the effect of nicotinamide in brain mitochondria was due to inhibition of NADase. However, this protective effect should become manifest only after a period of incubation when the concentration of pyridine nucleotides in the reaction mixture reached limiting concentrations because of NADase activity. Previous experiments[6] have established that under the conditions of incubation identical to those employed in these studies, maximum aerobic oxidation of isocitrate occurred when the level of added pyridine nucleotides was in excess of 2.0 umoles per 3 ml. of the reaction mixture. It is seen from Figure 1 that even in the absence of nicotinamide adult brain mitochondria oxidized isocitrate at a linear rate throughout the incubation period (40 min.) when the initial concentration of added pyridine

nucleotide was 3.0 umoles of NAD or NADP per 3.0 ml. reaction mixture. The influence of nicotinamide on both NAD and NADP mediated oxidations was evident at the commencement of incubation and this was maintained during the entire incubation period.

Table II shows the effect of nicotinamide on the oxidation of isocitrate and the concomitant oxidative phosphorylation in both brain and liver mitochondria. In the absence of added pyridine nucleotides, nicotinamide enhanced oxygen uptake only slightly, and it had no effect on oxidative phosphorylation in either liver or brain mitochondria. In the presence of added NAD, nicotinamide increased the oxygen uptake but it decreased oxidative phosphorylation in both tissues. When NADP was present, nicotinamide had a reverse effect on brain mitochondria, that is, it decreased the oxygen uptake and enhanced the oxidative phosphorylation. In a comparable incubation, nicotinamide decreased the oxygen uptake by liver mitochondria without affecting oxidative phosphorylation.

Oxidation in aged mitochondria

Addition of nicotinamide to aged mitochondria from either brain or liver increased the NAD dependent oxidation of isocitrate and depressed the NADP dependent oxidation, and thus it had an effect similar to that noted with intact mitochondria. The effect of nicotinamide on oxidative phosphorylation in aged mitochondria was not tested since in such

preparations phosphorylation is largely uncoupled from oxidation[9].

Isocitric dehydrogenases

Nicotinamide enhanced the NAD-isocitric dehydrogenase activity at all levels of mitochondria tested (Table IV). It had no effect on NADP-isocitric dehydrogenase activity at low concentrations of mitochondria (50 ug. protein per ml.), but it depressed this activity at higher mitochondrial concentrations (100-200 ug protein per ml.).

Cytochrome C reductases

Nicotinamide had no effect on NADH-cytochrome C reductase activity at low mitochondrial concentrations (20 ug protein per ml.), but it inhibited this enzyme activity when the level of mitochondria in the reaction mixture was increased (50-100 ug protein per ml.) (Table V). The NADPH-cytochrome C reductase was not appreciably affected by nicotinamide. When both NADH and NADPH were present, the total amount of cytochrome C reduced was much less than the sum when each of the pyridine nucleotides was present individually, and this reduced value was not further affected by added nicotinamide. Jacobson and Kaplan[4], and Vignais and Vignais[10] have also reported similar inhibitory effects of nicotinamide on the two cytochrome C reductases in liver mitochondria.

DISCUSSION

Nicotinamide at a concentration of 0.06 M enhanced the aerobic oxidation of isocitrate in both intact and aged mitochondria from adult brain and liver, but it had no effect on oxygen uptake by neonatal brain mitochondria. Since NADase is primarily an enzyme of adult tissue[8, 11], the function of nicotinamide appears to be concerned with its effect on this enzyme. A closer examination of the data shows that the observed effect of nicotinamide cannot be fully accounted for on this basis alone, since pyridine nucleotides were added in sufficient amounts to maintain a linear oxygen uptake throughout the experimental period (Figure 1). Also, nicotinamide increased the rate of oxygen uptake even at the start of incubation. In contrast to its action on NAD, nicotinamide inhibited the NADP mediated oxidation of isocitrate in both liver and brain mitochondria. If protection of pyridine nucleotides from NADase was the only function of nicotinamide, it should effect NADP as well as NAD, since both coenzymes are hydrolyzed by NADase[12].

Jacobson and Kaplan[13] found that liver homogenates with 0.05 M nicotinamide contained high levels of both oxidized and reduced pyridine nucleotides, suggesting that nicoeinamide not only inhibited NADase, but depressed the reoxidation of reduced pyridine nucleotides. Bassham and Birt[14] noted that in rat liver and pigeon mitochondria, 0.05 M

nicotinamide prevented the reduction of these coenzymes by endogenous substrates, but not by an externally added substrate. These findings indicate that in addition to inhibition of NADase, nicotinamide affects two types of enzyme reactions, one which reduced pyridine nucleotides and the other which oxidized the reduced forms.

It is seen from data presented in this report that nicotinamide enhances the activity of NAD-isocitric dehydrogenase, inhibits the activities of NADP-isocitric dehydrogenase and NADH-cytochrome C reductase, and has no effect on NADPH-cytochrome C reductase (Tables IV and V). All these enzymes, like NADase, depend for their activity on the binding of pyridine nucleotides, hence it is probable that nicotinamide acts competitively for the binding sites in these enzymes.

The mode of action of nicotinamide on oxidative phosphorylation in these studies is not clear due to lack of detailed information on the role of pyridine nucleotides in oxidative phosphorylation mechanisms. Griffith[15], and Griffith and Chaplain[16] have recently obtained some evidence for the existence of a labile intermediate in oxidative phosphorylation which contains equivalent amounts of NAD and phosphate. On the basis of the spectral changes that occur on hydrolyses of the intermediate, coupled with the fact that one of the products of the reaction is similar to the 'hydrated' form of $NADH_2$[17], they suggest

that the formation of this compound involved opening of the nicotinamide ring. It is possible that the action of nicotinamide noted in the present study can be attributed to its effect on the formation or breakdown of this high energy intermediate, due to blocking of enzyme sites concerned in these reactions.

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TABLE I

Effect of Nicotinamide on Isocitrate Oxidation
in Intact Brain Mitochondria

The reaction mixture contained 15 umoles $MgCl_2$, 2 umoles $MnSO_4$, 30 umoles KF, 5 umoles ATP, 0.04 umoles cytochrome C, 60 umoles glucose, 140 K.M. units hexokinase, 40 umoles DL-isocitrate, 40 umoles phosphate buffer (pH 7.4) and mitochondria equivalent to 0.5 gm. fresh brain in a total volume of 3 ml. When indicated in the table, 180 umoles of nicotinamide and 3.0 umoles each of pyridine nucleotides were added. Isocitrate, glucose, and hexokinase were transferred from the side arm after 7 minutes equilibration. Incubation, 40 minutes at 30°C.

	Experiment I Neonate		Experiment II Adult	
Coenzymes	Isocitrate	Isocitrate +NAm	Isocitrate	Isocitrate +NAm
	(ul O_2 uptake per flask)			
None	12.7	13.0	26.8	38.2
+ NAD	41.8	43.8	58.5	70.2
+ NADP	22.3	21.5	42.2	36.5
+ NAD + NADP	70.1	68.5	115	126

TABLE II

Effect of Nicotinamide on Isocitrate Oxidation and Phosphorylation in Intact Brain and Liver Mitochondria

Composition of the reaction mixture was the same as in Table I. Incubation, 40 minutes at 30°C.

	No coenzyme		NAD		NADP	
	O_2 (uatoms)	P:O	O_2 (uatoms)	P:O	O_2 (uatoms)	P:O
Experiment I: Brain						
Isocitrate	3.6	1.7	6.2	1.9	5.2	1.32
Isocitrate + NAm	4.2	1.8	7.3	1.4	4.0	1.75
Experiment II: Liver						
Isocitrate	3.5	2.1	6.2	2.3	4.8	2.0
Isocitrate + NAm	4.0	2.0	7.6	1.8	4.0	2.0

TABLE III

Effect of Nicotinamide on Isocitrate Oxidation in Aged Brain and Liver Mitochondria

Composition of the reaction mixture was the same as in Table I. Incubation, 40 minutes at 30°C.

	Experiment I Brain		Experiment II Liver	
Coenzymes	Isocitrate	Isocitrate +NAm	Isocitrate	Isocitrate +NAm
	(ul O_2 uptake per flask)			
None	8.5	12.2	12.3	18.5
+ NAD	50.7	67.0	90.5	118
+ NADP	35.5	22.3	75.0	55.2
+ NAD + NADP	67.8	76.4	147	121

TABLE IV

Effect of Nicotinamide on Isocitric and Malic Dehydrogenases in Aged Brain Mitochondria

Composition of the reaction mixture for measurement of isocitric dehydrogenase activity was as follows: 15 umoles DL-isocitrate, 0.8 umoles pyridine nucleotide, 50 umoles Tris (pH 7.3), 0.7 umoles of $MnCl_2$, 60 umoles of nicotinamide when added, and mitochondria as indicated to a total volume of 1 ml. Optical density recorded at 340 mu against a blank containing all reactants except pyridine nucleotides, in a 1 cm. cell at 30°C.

	Mitochondrial protein (ug)			
	20	50	100	200
	(mumoles of pyridine nucleotides reduced per minute)			
Isocitrate + NAD	10.2	18.7	21.0	14.0
Isocitrate + NAD + NAm	16.7	23.7	37.5	23.0
Isocitrate + NADP	--	39.7	78.5	157
Isocitrate + NADP + NAm	--	40.8	65.5	107

TABLE V

Effect of Nicotinamide on Cytochrome C Reductase Activity in Aged Brain Mitochondria

Composition of the reaction mixture: 0.5 umoles reduced pyridine nucleotide, 0.1 umole oxidized cytochrome C, 1.0 umole KCN, 75 umoles Tris (pH 7.3), 60 umoles nicotinamide when added, and mitochondria as indicated to a total volume of 1.0 ml. The blank contained all components except the pyridine nucleotides. Change in optical density at 550 mu recorded in a 1 cm. cell at 30°C.

	Mitochondrial protein (ug)			
	20	50	100	200
	(mumoles cytochrome C reduced per minute)			
NADH	14.3	28.5	51.5	-
NADH + NAm	14.3	24.8	42.0	-
NADPH	-	1.2	2.1	2.1
NADPH + NAm	-	1.0	2.6	1.9
NADH + NADPH	-	14.3	21.0	-
NADH + NADPH + NAm	-	14.8	23.0	-

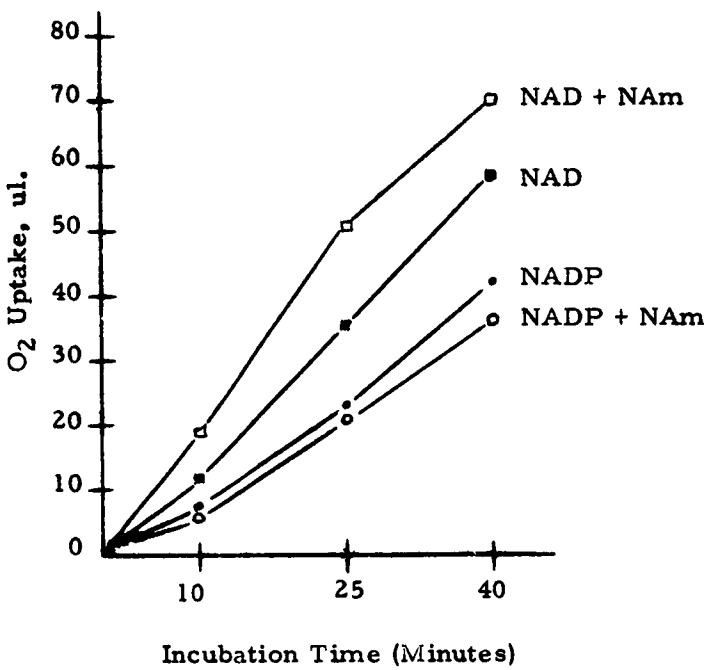

Fig. 1. Effect of nicotinamide on the oxidation of isocitrate by rat brain mitochondria. Composition of the reaction mixture was the same as that described in Table I.

METABOLIC CHANGES INDUCED IN MAMMALIAN ERYTHROCYTES BY WHOLE-BODY X-IRRADIATION

By Prof. D. A. RAPPOPORT and B. W. SEWELL

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APPLICATION of X-rays and radium in medicine, after their discovery during 1895–96, established their effectiveness in diagnosis and treatment of disease. Only somewhat later were the lethal and injurious properties of these penetrating rays recognized[1]. Since then, large groups of men have become exposed more frequently to man-made radiation. These additional exposures to penetrating radiation have magnified the need for a reliable indicator of radiation-induced tissue damage. What is precisely needed is a simple and accurate indicator which would correlate biological damage with the radiation dose[1].

The requirements of an ideal biological radiation indicator are that : (*a*) a tissue or tissue component should show changes over extended periods following whole-body irradiation ; (*b*) this change can be quantitatively measured. It is also important that tissue samples should be available for intermittent sampling without injury to the subject and without alteration in the system under examination.

Choice of Erythrocytes

Certain generalizations can be used in considering this problem in order to initiate a working hypothesis. Tentatively it can be assumed that any radiation absorbed by cells will cause changes in the cell enzymes[2], but that the detection of these changes is dependent on (*a*) the sensitivity to radiation of a particular enzyme system under evaluation and (*b*) the degree of sensitivity of the analytical methods employed.

Implicit in the above specifications is the fact that the tissue must be incapable of extensive internal repair if it is to reflect any post-irradiation changes. This immediately eliminates tissues with large populations of mitotic cells and suggests erythrocytes as the tissue component of choice. In man the erythrocyte has a life-span of 110–120 days[3], in the rat this span

is 49–55 days[4], in other mammals erythrocyte life-spans are between these values[5]. Since mammalian erythrocytes are enucleated, no resyntheses of proteins can occur, and any radiation damage incurred on the enzyme-proteins, such as denaturation or rupture of peptide linkage, should be detectable by changes in enzymic reactions. This rationale suggests the erythrocyte enzymes for examination as a test system.

If the hypothesis is held that any absorbed radiation will affect enzymes in all cells, how is it that no enzymic changes have been observed in erythrocytes after moderate whole-body irradiation? This may be explained on the basis that up to the present time few erythrocyte enzymes have been tested after radiation treatment. Erythrocyte enzymology has now been more thoroughly explored[6,7]. With the complete elucidation of glycolysis, the hexosemonophosphate shunt, the transketolase and transaldolase enzymes, and nucleoside phosphorylase in erythrocyte extracts, re-examination of the radiation effect on these enzyme systems is in order.

Nucleoside Metabolism

Investigators concerned with the preservation of blood have found that when inosine or adenosine is added to blood the integrity of the erythrocytes is maintained during storage and their survival follow-

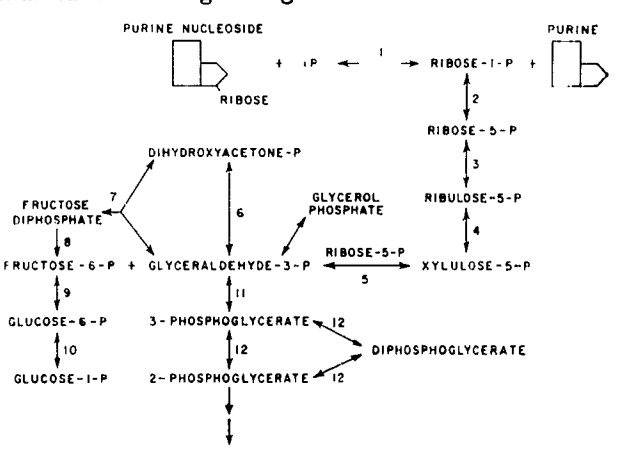

Fig. 1. Diagrammatic representation of the metabolic reactions of purine nucleosides in erythrocytes. The enzymes involved are: (1) nucleoside phosphorylase; (2) phosphoribomutase; (3) phosphoriboisomerase; (4) phosphoketopentoepimerase; (5) transketolase; (6) triosephosphate isomerase; (7) aldolase; (8) fructose diphosphatase; (9) phosphoglucoisomerase; (10) phosphoglucomutase; (11) triosephosphate dehydrogenase; (12) phosphoglyceric mutase

ing transfusion is improved[5]. This was attributed to the resynthesis of metabolites essential for erythrocyte integrity.

The enzymes responsible for nucleoside metabolism within the erythrocyte are in the soluble portion of the cell. The reactions which they catalyse are diagrammatically illustrated in Fig. 1. First, the nucleoside in presence of phosphate is converted to ribose-1-phosphate and a purine base by a nucleoside phosphorylase[6]. Later, the pentose phosphate is transformed to a variety of phosphate esters via the actions of transketolase, transaldolase and the glycolytic enzymes[6].

When considering nucleoside metabolism in erythrocytes as a system for evaluation of X-ray effects, it can be assumed that if any enzyme among the group of interdependent reactions is inhibited there will be an accumulation of substrate and a change in the yield of phosphate esters. Complex multiple enzyme systems, such as are involved in erythrocyte metabolism of nucleosides, have certain disadvantages as well as advantages in studies of radiation effects. The disadvantage in such systems is that it may be difficult to determine which particular enzyme was affected by the radiation. However, the advantage in using a multiple enzyme system is that this increases the opportunity of finding one or more enzymes sensitive to absorbed radiation.

Erythrocyte Turnover

In the studies on the enzymic changes at prolonged post-irradiation intervals, time of residence of the circulating erythrocyte is a major factor for consideration. This requires that the maximum post-irradiation time-interval used for evaluation must be within the period when the irradiated erythrocyte population is not markedly altered. This can be calculated from the erythrocyte life-span.

Inbred strains of rats are convenient for radiological studies. The 'mean life-span' of rat erythrocytes as well as the 'half-clearance time' can be used to estimate changes in erythrocyte population. The 'mean life-span' of an erythrocyte is the average interval of time any erythrocytes will remain in circulation; 'half-clearance time' is that time-interval at which 50 per cent of the circulating erythrocytes will disappear from circulation. Belcher and Harriss[4] have recently reported the 'mean life-span' of the rat erythrocyte as 49–55 days. The 'half-clearance time' was determined as $20{\cdot}7 \pm 2{\cdot}5$ days.

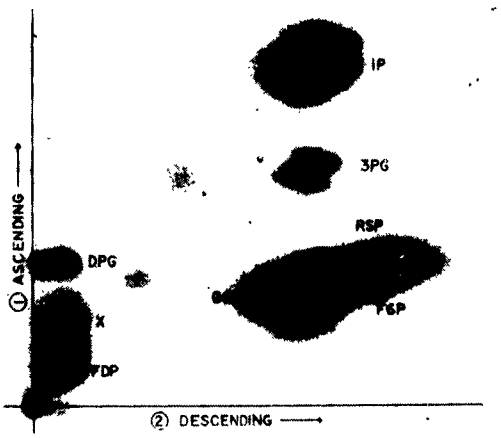

Fig. 2. Radioautogram of phosphate esters separated by two-dimensional paper chromatography, (1) ascending with ethyl-acetate, acetic acid and water (3 : 3 : 1) and (2) descending with methyl ethyl ketone, methyl cellosolve, ammonia and water (7 : 2 : 0·7 : 2·3). *iP*, inorganic phosphate; *3PG*, 3-phosphoglycerate; *R5P*, ribose-5-phosphate; *G6P*, glucose-6-phosphate; *DPG*, 2,3-diphosphoglycerate; *X*, unidentified ester; *FDP*, fructose diphosphate

Since these observations are based on the mean life-span and half-clearance time of erythrocytes in the normal rats, it is important to know how these figures are altered in X-irradiated rats. Total-body X-irradiation of rats in excess of 300 r. causes complete inactivation of marrow and stops the extrusion of erythrocytes into circulation[10]. However, the life-span and half-clearance time of the circulating erythrocytes remain relatively the same as in the unirradiated animals[11]. There is evidence that random (non-senescent) destruction of erythrocytes, which in the normal rat is approximately 0·48 per cent per day[5,6], is increased in the irradiated animals but the magnitude of this change is unknown[11].

Recognizing the prolonged inactivation of marrow after an X-ray dose of 300 r. or higher, we see that the erythrocyte population will consist only of irradiated cells up to and even beyond a two-week period, since no new cells are extruded by marrow during this time. However, due to internal hæmorrhages and other undefined factors, random non-senescent loss of erythrocytes will be increased[11]. This will cause a drop in the number of red cells; however, loss of plasma into tissue and dehydration due to vomiting and diarrhœa will tend to decrease plasma volume, hence there may be no net change in hæmatocrit values[11].

The above discussion can be summarized by the

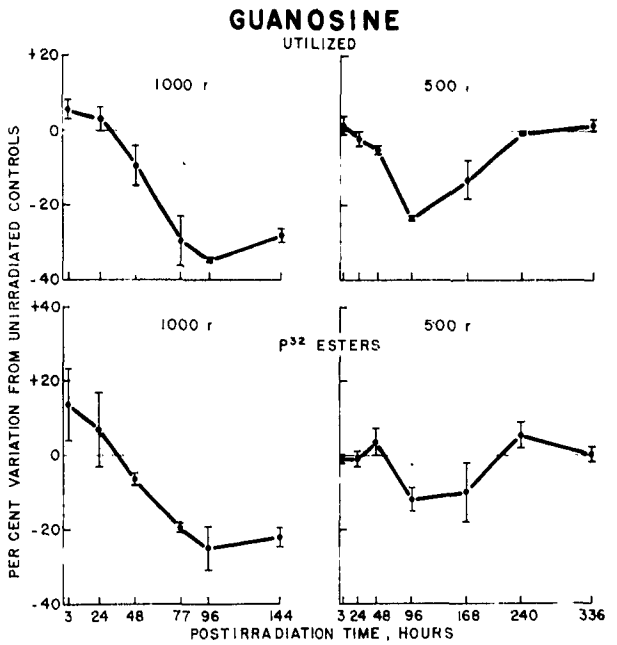

Fig. 3. Comparative utilization of guanosine and formation of phosphate esters by rat erythrocyte extracts from rats irradiated with 1,000 and 500 r. X-rays, respectively

conclusion that following total-body radiation with doses at and above 300 r. the erythrocyte population remains relatively undiluted and the life-span and half-clearance time remain approximately the same as in the unirradiated rat, with the exception that there is an increased random loss of erythrocytes which cannot be quantitatively evaluated at present.

It is tentatively concluded that the erythrocyte is a promising tissue component as an internal mammalian X-ray indicator, since: (*a*) it has a wide variety of enzymes among which some may be sensitive to radiation; (*b*) it cannot replace altered enzyme-proteins; (*c*) it has a long life-span even in irradiated animals; (*d*) it is accessible for repeated sampling.

The concepts discussed above were tested by measuring the changes in nucleoside metabolism by erythrocyte extracts from irradiated and unirradiated rats. Our results established that following 1,000 r. whole-body X-irradiation the utilization of purine nucleosides was markedly depressed up to 144 hr. after irradiation. Whole-body irradiation of 500 r. also depressed purine nucleoside metabolism for a period of 96 hr., when the metabolism began to in-

crease and by 240 hr. reached the level of substrate utilization by erythrocyte extracts from unirradiated controls. These results establish that erythrocyte enzymes are affected by moderate whole-body radiation and that these changes are detectable over a period from one to two weeks after irradiation.

Experimental Results

Erythrocytes from irradiated and unirradiated Sprague–Dawley rats were separated from white cells and plasma, lysed in water, dialysed overnight; extracts were then prepared by centrifugation. These extracts were incubated for 2 hr. in *tris* buffer at *p*H 7·4 with either guanosine, inosine, or adenosine in the presence of inorganic phosphate labelled with phosphorus-32 and magnesium chloride. The detailed procedures and techniques will be described elsewhere[12]. After incubation, the remaining purine nucleoside was analysed, total organic phosphate was determined, and the nature of the individual phosphate esters formed was established by means of paper chromatography[13].

The reactions studied in these incubations are represented schematically in Fig. 1 and the phosphate esters actually formed from guanosine and labelled inorganic phosphate are shown by the radioautogram in Fig. 2. The same phosphate esters were also obtained from incubations of inosine and adenosine. Enzyme activity of erythrocyte extracts from rats irradiated with 1,000 r. or 500 r. to the whole body were compared with extracts from unirradiated controls. A decrease in substrate utilization and phosphate ester formation was observed. The results of these experiments are illustrated in Figs. 3, 4 and 5.

Erythrocyte extracts from rats treated with 1,000 r. showed enhanced utilization of guanosine up to 24 hr. after irradiation (Fig. 3); but afterwards both guanosine utilization and phosphate ester formation decreased and at 96 hr. reached a minimum value and remained at this level until the death of the animals in 7–8 days. Extracts from the rats treated with 500 r. showed a decrease in guanosine utilization in 24 hr. after irradiation and this reduced enzyme activity continued until the ninety-sixth hour (Fig. 3; these are similar to the results with the 1,000 r. extracts). Afterwards, the utilization of guanosine increased again and in 240 hr. the level of substrate utilization was equal to that of the controls. Almost identical results were obtained with inosine, as shown in Fig. 4. However, utilization of inosine by erythrocyte

extracts from 500 r. treated rats did not reach the level of inosine utilization by the controls until 336 hr. after irradiation. Experiments with adenosine utilization showed results similar to those of inosine.

Significance of Results

These experiments establish that erythrocyte enzymes are affected by lethal (1,000 r.) and sub-lethal (500 r.) whole-body X-irradiation. Also the inhibitory effect of radiation persists for seven days or longer depending on the X-ray dose. Since the same enzymes are involved in the metabolism of guanosine, inosine or adenosine, one would expect radiation to influence these reactions in a similar way, as they did (Figs. 3, 4 and 5).

Although nucleoside metabolism by the erythrocyte extracts from rats treated with 1,000 r. was inhibited during their survival (7–8 days), the extracts from the rats treated with 500 r. X-rays showed a marked inhibition for the initial interval up to 7 days after radiation. This was followed by the recovery of enzyme activity by 7–10 or 14 days post-irradiation. This recovery of activity suggests that with 500 r. whole-body radiation the inhibition was not due to

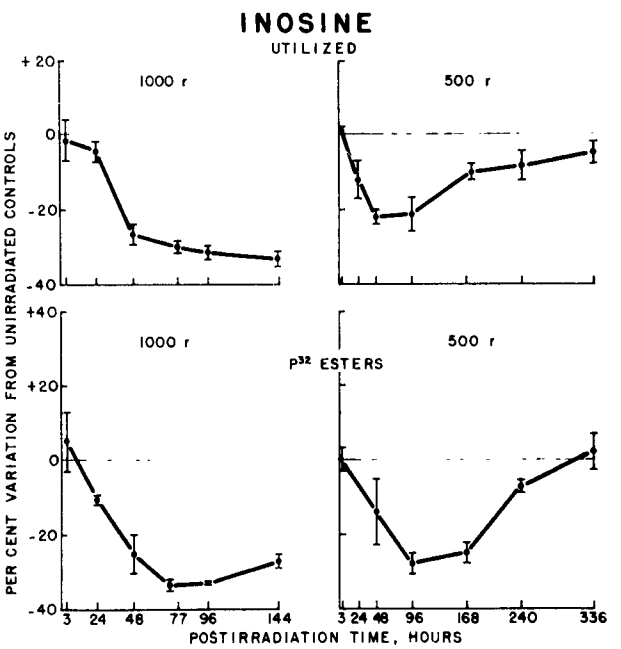

Fig. 4. Comparative utilization of inosine by rat erythrocyte extracts

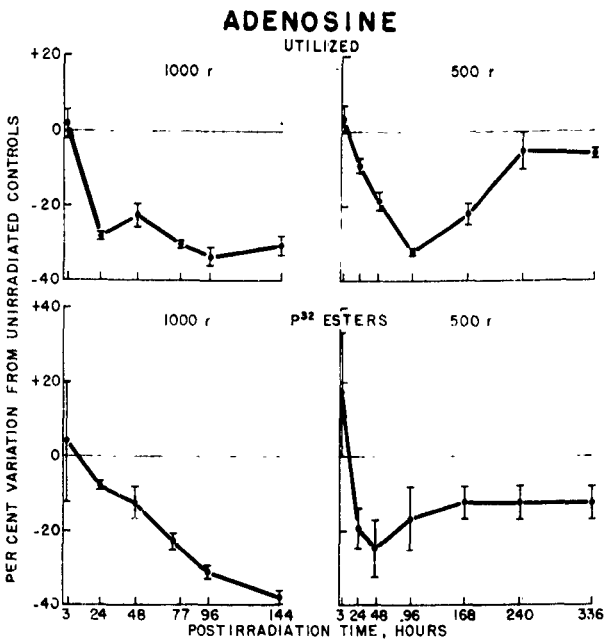

Fig. 5 Comparative utilization of adenosine by rat erythrocyte extracts

irreversible protein damage, but probably to the oxidation of some essential sulphydryl groups to disulphides. Since the erythrocytes contain glutathione, it is likely that the disulphide groups were gradually reduced by the glutathione during the period of recovery of enzyme activity.

Although the radiation does affect the erythrocyte metabolism, how useful is this information in evaluating erythrocyte metabolism as a biological radiation indicator ? Data from Figs. 3, 4 and 5 also show that the degree of inhibition, particularly for guanosine and inosine metabolism (Figs. 3 and 4), is appreciably greater at 48 hr. after 1,000 r. irradiation than after 500 r. treatment. Adenosine metabolism decreased in 24 hr. to a greater extent in the rats treated with 1,000 r. than in those receiving 500 r. The variations in erythrocyte activity described above following 1,000 r. and 500 r. irradiation are only the initial observations. Additional data will be necessary to establish the relationship between whole-body radiation dose and degree of enzyme inhibition in erythrocyte extracts. The present results suggest that the metabolism of nucleosides following radiation can serve as a gross biological indicator. This information may encourage other investigators to examine the

erythrocyte metabolism in other species, particularly cancer patients requiring total-body radiation treatment, in order to ascertain whether X-ray induced inhibition of rat erythrocyte metabolism also occurs in other animals, as well as in man.

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